OPTIMIZATION AND VALIDATION OF A MULTIPLEX PCR FOR IDENTIFICATION OF MAMMALIAN BLOOD MEALS IN MALARIA VECTOR MOSQUITOES AND TIME-COST COMPARISON BETWEEN THE PCR AND ELISA METHODS

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Background

Blood meal identification for haematophagous insects, including malaria vector mosquitoes, is critical in understanding the vectorial capacity of species and populations. This is because the feeding behaviour of mosquitoes varies greatly, with some species feeding either exclusively on humans (anthropophily) or animals (zoophily), or both. As only those *Anopheles* species that take human blood are potential vectors of human malaria, identification of the blood meal source/s of species-identified female mosquitoes provides important information concerning malaria vector incrimination.

Methods for determining the blood meal source of haematophagous insects have evolved greatly over the years. Precipitin tests have been used to detect the blood meal source of insects for many decades.[1] In the 1980s, an enzyme-linked immunosorbent assay (ELISA) was introduced as a more sensitive alternative.[2] Both methods have their advantages and disadvantages and were often chosen based on the situation and level of accuracy, specificity and sensitivity required.[3] Other methods such as haemoglobin crystallization[4], agglutination reactions[5] and immunoflourescence[6,7] have either been proposed or used. Although each method has proved useful, they are either inadequately sensitive, inadequately specific, unreliable or too elaborate - requiring sophisticated equipment.[2]

The need for a blood meal identification method that is both specific and sensitive led to many laboratories adopting the ELISA method, which is currently the most widely used. However, the advancement of molecular techniques and the requirement to carry out multiple molecular diagnostics on single specimens led to the development of polymerase chain reaction (PCR) techniques for blood meal identification.[8] These methods are mainly based on cytochrome b, a well characterized protein from complex III of the mitochondrial oxidative phosphorylation system.[9,10] In order to reduce the cost and time involved, Kent & Norris[8] developed a multiplex PCR based on cytochrome b for mammalian blood meal identification in malaria mosquitoes. This method directly identifies mammalian blood in mosquitoes by the amplification of size-specific DNA fragments.

The aim of this study was to assess the reliability, cost effectiveness and efficiency of the multiplex PCR technique of Kent & Norris[8] in comparison to the routine ELISA method.[11]

Materials and Methods

Mosquito samples

Indoor resting mosquitoes were collected from six villages spanning two ecological zones (forest and coastal savannah) in Ghana during September to December, 2010. Immediately after collection, mosquitoes were dry preserved using silica gel. Each specimen was identified to species using morphological keys[12,13] and PCR[14,15] as appropriate.

DNA isolation

DNA was extracted[16] from the abdomens of all female mosquitoes. Abdominal DNA from a female mosquito fed on human blood served as a positive control. Other positive controls consisted of DNA extracted from whole cow, pig, goat and dog blood collected from the Department of Agriculture, University of Pretoria. The DNA pellets were re-suspended in 200 µl of 1 x TE Buffer and stored at -20°C until ready for use.

Optimization and validation of the blood meal identification PCR protocol

The blood meal PCR assay was initially implemented according to the protocol of Fornadel *et al.*[17] A hot start *taq* Polymerase is required for this protocol. The PCR reaction was adapted slightly with an annealing temperature of 58°C.

In an effort to reduce the overall cost per specimen, the PCR reaction mixture was halved to 12.5 µl reaction volume and a standard *taq* Polymerase was used. The optimised 12.5 µl reaction mixture consisted of: 1.25 µl 10x PCR buffer, 0.5 mM dNTP mix, 0.75 mM $MgCl_2$, 50 pmol of each primer (UnvRev 1025, Pig573F, Human894F, Goat368F, Dog368F, Cow121F), 0.5U Taq DNA polymerase and 0.5 µl template DNA. The following cycling conditions were used: 95°C for 5 minutes followed by 95°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute for 35 cycles with a final extension at 72°C for 7 minutes.

This optimized Kent & Norris[8] protocol was validated on 350 mosquito samples comprising 243 *Anopheles gambiae* and 107 *An. funestus* specimens. A single PCR amplicon for each blood type identified was sequenced to confirm the accurate identification of the blood meal source. This was accomplished by aligning the sequences obtained to known sequences for each blood source which are available on the NCBI database.

Cost and time comparison between PCR and ELISA

The cost of blood meal identifications using PCR and ELISA was generated in South African Rands (ZAR) and then converted to United States Dollars (USD) using the South African Reserve Bank exchange rate of 8.1897 ZAR : 1.0 USD for 11th July, 2012. These costs only included consumables, with the assumption that equipment is a basic prerequisite for running these assays. The costs were compiled from the suppliers quotes for bulk purchases (including VAT), and refined to cost per analysis of a 96 well plate for ELISA and a 36 well agarose gel for PCR. From these, the costs of supplies needed to analyse one sample for either ELISA or the multiplex PCR were determined.

The time required to conduct blood meal identification using either PCR or ELISA was derived based on a 36 well gel and a 96 well plate respectively. The following was taken into consideration when calculating the time required for PCR identifications: time required to extract DNA, prepare the PCR reaction mixtures, cycling time of the PCR reaction in the PCR machine, preparation of the agarose gel, electrophoresis and gel analysis. The following was taken into consideration when calculating the time required for ELISA identifications: time required to prepare the microtitre plates, process the mosquito homogenates, complete the incubation steps of the ELISA protocol and analyse the optical densities obtained for the ELISA protocol. Hands on time (active work on the process minus waiting times) and whole process time were estimated for both PCR and ELISA.

Results

Optimization and validation of the PCR protocol

Analysis for bloodmeal identification was based on the protocol of Fornadel *et al.*[17] with a change of annealing temperature from the recommended 56°C to 58°C Sequence analysis of selected blood meal PCR amplicons confirmed that the correct host, (human, cow, pig or dog) was amplified. PCR validation of 350 blood fed indoor resting mosquito specimens resulted in 82.3% and 94.3% successful blood meal identifications for *An. gambiae* and *An. funestus* respectively (table 1) based on amplicon sizes visualised by electrophoresis.

Table 1: Identification of blood meal sources of *Anopheles gambiae* and *An. funestus* females from Ghana using the optimized blood meal PCR protocol of Kent &Norris.[8]

Anopheles Spp.	n (%)	No. blood meals successfully identified (%)	Blood meal Source (%)		
			Human	Animal	Mixed
gambiae	243 (69.4)	200 (82.3)	188 (77.4)	1 (0.4)	11 (4.5)
funestus	107 (30.6)	102 (95.3)	102 (82.9)	0 (0)	0 (0)
Total	350	302 (86.3)	290 (96.0)	1 (0.003)	11 (3.6)

Time Cost Analysis for PCR and ELISA

Table 2 shows the time and cost analysis for PCR vs ELISA. The multiplex PCR method requires less time (8.78 hours) to identify five blood meal sources simultaneously, compared to ELISA which requires 23.75 hours for five blood meals assuming that these are performed one at a time. This time can be significantly reduced if all five assays are performed concurrently. PCR is more cost effective and currently costs approximately US $0.93 per five blood meal sources per sample as compared to US $3.19 for the equivalent identifications using ELISA.

Table 2. Comparative cost and time required for the PCR and ELISA methods for mosquito blood meal source identification. Hands on time refers to processing time without incubation periods included.

	Time to Complete (hrs)			
	Whole Process	Hands on	Cost per Sample	
Multiplex PCR for 5 Blood Meals	8.78	4.28	R 7.61	($ 0.93)
ELISA for 1 Blood Meal	4.75	0.25	R 5.22	($ 0.64)
ELISA for 5 Blood meals	23.75	1.25	R 26.10	($ 3.19)

Discussion

Laboratories that process large numbers of mosquito specimens for multiple diagnostic features require methods and processes that are high throughput, efficient and cost effective. Although the ELISA method is sensitive in terms of identifying the source of blood meals[2], it is time consuming, comparatively expensive and is limited in terms of throughput. The optimized multiplex PCR protocol[8] for blood meal identification is equally sensitive. The 13.7% of specimens from which blood meal source could not be identified, even after two repeat attempts, suggests that they may have taken blood meals from other domestic animals such as cats, sheep, chicken, duck, turkey or even peridomestic pests such as rats, mice and bats. Other factors such as DNA degradation, enzyme inhibitors and human error cannot be ruled out.

The multiplex PCR assay, compared to the ELISA method, appears to be quicker and less costly when assaying for several blood meal sources. However, if the aim of identifying blood meal source is only to determine the proportion feeding on humans, such as determination of the human blood index (HBI), then ELISA is a better option in terms of cost and time.

A major advantage of the multiplex PCR over ELISA is that the PCR can be integrated with other molecular diagnostic methods, especially in laboratories that conduct multiple diagnostics on single specimens using the extracted DNA of each specimen.[8] Under these circumstances, apart from tailored primers, all the re-

agents used for blood meal identification by PCR are universal for to any diagnostic PCR assay. Purchasing these reagents in large quantities can further reduce the costs for all of the PCR diagnostic processes employed. Furthermore, using single DNA extracts for multiple diagnostics should reduce the time required to prepare specimens and thus enhance throughput.

It is concluded that the multiplex PCR method of Kent & Norris[8] can significantly reduce the time required for and cost of identification of blood meal sources of mosquitoes and other haematophagous insects. This protocol can be adopted for routine blood meal source identification with ELISA retained as an alternative method in situations where only the human blood index is of interest.

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